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The New Serine-Threonine Kinase, Qik, Is a Target of the *qin* Oncogene

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The winged helix transcription factor Qin is the avian homolog of the mammalian brain factor 1 (BF-1) and has the potential to act as an oncogenic protein. We used representational difference analysis to identify genes that are differentially expressed in chicken embryo fibroblasts (CEF) transformed by Qin. One of the up-regulated Qin targets identified in this analysis is a serine-threonine kinase termed Qik (Qin-induced kinase). Qik belongs to the AMPK/SNF1 kinase family. It is a ubiquitously expressed protein and is upregulated rapidly after a hormone-regulated form of Qin is activated. *In vitro* kinase tests demonstrate that Qik is capable of autophosphorylation. Elevated levels of Qik transcripts are also observed in Src-transformed cells, suggesting that Src and Qin share some targets. © 2000 Academic Press

Key Words: **brain factor 1; differential gene expression; kinase; Src protein; oncogenic transcription factor; chicken embryo fibroblasts.**

The *qin* gene was isolated as the oncogenic determinant of the avian retrovirus ASV31; it codes for the avian homologue of the mammalian brain factor 1 (BF-1) (1, 2). The Qin protein belongs to the family of forkhead/winged helix transcription factors defined by a conserved 100-amino acid DNA-binding region, the winged helix domain. Forkhead/winged helix proteins have been recently renamed FOX proteins (for forkhead box) and given individual letter designations (3). Qin corresponds to FoxG1. Qin expression is restricted to the telencephalon and telencephalon-derived tissues (4). Its mammalian counterpart, BF1, is essential for the development of the forebrain. Mouse null mutations of BF-1 die at birth, showing a drastic reduction in the size of the cerebral hemispheres, that probably results from premature onset of neuronal differentiation and depletion of the pool of neuroepithelial progenitor cells (5). Injection of *qin* mRNA into Xenopus embryos leads to suppression of neurogenesis, suggesting that Qin acts as negative regulator of neuronal differentiation (6). Overexpression of Qin in the developing chicken embryo causes a disturbance of the visual projection map on the optic tectum because of aberrant neuronal guidance (7). In cultures of chicken embryo fibroblasts (CEF), Qin induces oncogenic transformation; it also causes tumors in the wing web of young chickens (1). Qin can function as a transcriptional repressor, binding the DNA consensus sequence TGTAAACAAA (8). Repression activity is correlated with the oncogenicity of the Qin protein (9). Target genes that are controlled directly by Qin in transformed cells would therefore be expected to show reduced expression. Indirect targets, on the other hand, could also be upregulated. We used representational difference analysis to conduct a systematic search for genes that are differentially expressed in Qin-transformed CEF. Among the potential Qin targets identified in this fashion is the cDNA clone of a gene that shows significantly elevated expression in transformed CEF. It codes for a new member of the AMPK/SNF1 family of serine/threonine kinases.

MATERIALS AND METHODS

Plasmid construction. The construct RCAS-Qin-ER, allowing estrogen-dependent regulation of Qin activity, was made by digesting pBK-v-Qin (9) with EspI, filling overhangs with Klenow polymerase and fusing it in-frame to the hormone-binding domain of human estrogen receptor (ER), which was excised from the HE14 plasmid (10) by cleavage with BamHI and mung bean nuclease followed by EcoRI digestion. The v-Qin-ER insert was then subcloned into the BamHI-EcoRI sites of the adapter plasmid CLA12Nco and moved into the avian retroviral expression vector RCAS (11). The RCAS constructs expressing the oncogenes *src*, *jun*, *qin* and *p3k*, as well as RCAS-v-Qin-VP16 and RCAS-v-Qin-En have been described previously (9, 12–14).

The following PCR primers were synthesized to construct pBFI-Qik: qikI: 5′-CAA GGA TCC GCC ATG GTG TAT CCT TAC GAT GTA CCA GAC TAT GCG ATC ATG TCG GAG TTC GCC TCC-3′,

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and qik2: 5'-TGT CTC CAT AAC CTG ATA CAG CTT G-3'. In the primer qik1, a hemagglutinin tag sequence YDYDVPDYA was inserted after the ATG start codon to facilitate immunological detection. The amino terminus of Qik amplified by PCR using qik1 and qik2 primers was digested with BamHI and HindIII and cloned into the pBSFI adapter plasmid (15) together with a HindIII-MluI fragment which covers the rest of Qik coding region to generate pBSFI-Qik. For the construction of the kinase-negative mutant, Qikm, carrying a K to M substitution at position 55, the BamHI-HindIII fragment from pBSFI-Qik was first cloned into the pBSFI vector, and mutants were created on this template using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the following primers: qikm1: 5'-CTT GCC ATA ATG ATA ATA GAC AAA ACG-3', and qikm2: 5'-CGT TTT GTC TAT TAT TTT TAT GGC AAC-3'. After sequence analysis, the BamHI-HindIII region in pBSFI-Qik was replaced by the mutated counterpart to generate pBFI-Qikm. The inserts from all pBSFI adapter plasmids were subsequently excised with the SfiI restriction enzyme and cloned into the RCAS expression vector.

RNA preparation and Northern blot analysis. Poly(A)$^+$ RNA was isolated from CEF using the RNA-STAT-60 kit (TEL-TEST, Inc., Friendswood, TX) and the Oligotex mRNA kit (QIAGEN, Valencia, CA). cDNA probes were labeled with [α-^{32}P]dCTP using the random primer labeling kit (Boehringer Mannheim, Indianapolis, IN).

Subtractive hybridization of cDNA. The PCR-Selected cDNA Subtraction Kit (Clontech, Palo Alto, CA) was used for detection of differentially expressed genes in Qin-transformed CEF according to the manufacturer's instructions. The technique is known as cDNA-based representational difference analysis (RDA). mRNA (2 μg) from CEF infected with the empty retroviral expression vector RCAS was used as driver, and the same amount of mRNA extracted from RCAS-v-Qin infected CEF was used as tester. This driver/tester combination identifies mRNAs that are overexpressed in RCAS-v-Qin-infected CEF. cDNAs from both mRNA populations were hybridized using two-fold excess of driver sample. Subtracted cDNAs were amplified by PCR and cloned into the pGEM-T vector (Promega, Madison, WI).

cDNA and genomic library screening. 1×10^6 phages from a cDNA library of ASV31-infected CEF were screened following standard protocol (16). The 800 bp RDA product representing *qik* cDNA was used as a probe for plaque hybridization. Positive plaques were selected and amplified on 15 cm agar plates. Phage DNA was prepared with the phage DNA kit (QIAGEN, Valencia, CA) for subcloning and sequencing. In order to obtain the sequence that covers the start codon of Qik, 2×10^5 phages from a chicken genomic library were also screened, and clones were analyzed as described above.

Cell culture and transfection. Primary CEF were prepared and cultured according to published techniques (17). Cells were transfected with plasmid DNA and LipofectAmine (Gibco BRL, Grand Island, NY) following the manufacturer's instructions. The transfected CEF were transferred three to four times to allow spread of the infection to all cells before they were used for kinase tests or focus enhancement assays.

Immunofluorescence. CEF infected with RCAS-Qin-ER were seeded on coverslips at a density of 2×10^4 cells per 36 mm culture well and incubated for 24 h. Cells were treated with 200 nm 4-hydroxytamoxifen (4-HT) (Research Biochemicals International, Natick, MA) or ethanol and fixed in phosphate-buffered saline containing 3.7% formaldehyde. Nuclear staining of the v-Qin-ER chimeric protein was detected using a Qin polyclonal antibody (1:100) and FITC conjugated anti-rabbit IgG (1:100) (Sigma, St. Louis, MO).

Immunoprecipitation, in vitro kinase assay, and immunoblotting. RCAS-Qik or RCAS-Qikm-infected CEF were collected, washed with phosphate-buffered saline and lysed in NP-40 lysis buffer. Cellular debris was removed by centrifugation at 16,000*g* for 15 min at 4°C, and the Qik protein in the supernatant was immunoprecipitated by incubating of 100 μg protein from the cell lysate with an anti-HA monoclonal antibody (12CA5, Covance, Richmond, CA) at 1:500 dilution. For *in vitro* kinase assays, immunoprecipitated protein was incubated with [γ-^{32}P]ATP at 30°C for 20 min in kinase assay buffer. Samples were denatured at 100°C for 5 min and analyzed on a SDS–PAGE gel followed by transfer to a nitrocellulose membrane. Phosphorylation was detected by autoradiography. Protein samples from the cell lysates were also separated on SDS–PAGE for immunoblotting. The blot was stained with the monoclonal anti-HA antibody 12CA5 at a dilution of 1:2000 and a horseradish peroxidase-conjugated anti-mouse serum at a dilution of 1:3000 (Amersham Life Science, Piscataway, NJ). The kinase substrates histone H1, H2b were bought from Boehringer Mannheim (Indianapolis, IN), and cdc25 was obtained from Dr. Steven Reed (The Scripps Research Institute, La Jolla, CA).

RESULTS

Isolation of qik as a gene that is up-regulated in qin-transformed CEF. In a search for potential target genes of the Qin protein, cDNA from CEF infected with RCAS was used as driver and cDNA from CEF transformed by RCAS-v-Qin as tester in cDNA-based RDA. After two rounds of subtractive hybridization, an aliquot of the PCR products was resolved on a 1% agarose gel. A distinct band of 800 bp was identified, provisionally termed OV4; it was cloned and sequenced. A database search did not reveal significant homology of OV4 to known sequences present in GenBank. Northern blots confirmed increased levels of OV4 mRNA in Qin transformed CEF. A cDNA library derived from Qin-transformed CEF was then screened for clones containing the complete coding region of OV4. Ten positive clones with overlapping open reading frames were isolated, subcloned and sequenced. The clone with the longest insert, about 5 kb, was chosen for further analysis. This cDNA fragment, used as a probe in Northern blots of mRNA from CEF, detected a major band at 5.1 kb and two minor bands at 4.4 kb and 4 kb (Fig. 1). The minor bands may represent products of alternative splicing. The levels of all three RNA species, particularly the major one, were found significantly enhanced in both RCAS-c-Qin and RCAS-v-Qin-transfected CEF compared to the RCAS control (Fig. 1). A similar result was obtained with RCAS-v-Qin-En, a chimeric plasmid constructed by fusing the DNA binding domain of Qin with the repression motif of the Drosophila Engrailed protein (9). As an additional control, the chimeric transcriptional activator RCAS-v-Qin-VP16 was also included. In this construct, the repression domain of v-Qin is replaced with the transactivation domain of the herpes simplex virus protein VP16 (9). In contrast to the wild type Qin and Qin-En constructs, expression of v-Qin-VP16 resulted in the suppression of OV4 transcripts (Fig. 1).

Qik belongs to the AMPK/SNF1 family of serine-threonine kinases. The deduced amino acid sequence of the 5 kb OV4 cDNA clone shows significant homology to the catalytic domains of the AMPK/SNF1 family

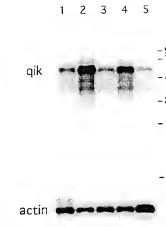

FIG. 1. Up-regulation of Qik mRNA in Qin-transfected CEF. Poly A+ RNA from cells transfected with the RCAS vector alone (lane 1), RCAS-c-Qin (lane 2), RCAS-v-Qin cloned into anti-sense orientation (lane 3), RCAS-v-Qin (lane 4), and RCAS-v-Qin-VP16 (lane 5) was separated by electrophoresis, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled Qik cDNA probe. Size markers are shown on the right. Expression of *c-qin* or of *v-qin* results in increased levels of *qik* mRNA, antisense *qin* has no effect on *qik* mRNA levels and *qin-VP16* reduces the levels of *qik* mRNA.

of kinases, suggesting that OV4 may code for a functional protein kinase (Fig. 2). This potential target gene of Qin was therefore named *qik*, for *qin*-induced kinase. The amino-terminal region of the Qik protein contains 11 kinase subdomains that are conserved in other serine-threonine protein kinases. The carboxyl-terminal part does not show significant homology to known sequences except for a similarity to the gene of salt-inducible kinase (SIK), which was cloned recently from the rat adrenal gland (18). The homology between Qik and SIK extends to both catalytic (97%) and non-catalytic domains (68%). Qik may therefore share functional specificities with SIK, possibly regulating ion channels. Since the 5 kb Qik cDNA clone did not contain a start codon, a chicken genomic library was screened with a probe that included the most 5′ region of the cDNA clone. A genomic clone was identified, that contains a start codon with the conserved Kozak sequence followed by coding information that is in-frame with the downstream sequence derived from the truncated Qik cDNA clone. The existence of a stop codon immediately upstream of the Kozak sequence excludes the possibility that the translation of the Qik protein is initiated from ATG sequences further upstream. In order to verify that the 5′-sequence of Qik obtained from the genomic clone is present in the Qik mRNA, RT-PCR was carried out using total RNA from CEF as the template with a primer in the presumptive upstream untranslated region identified in the genomic clone and a second primer within the coding region of the truncated cDNA clone. This RT-PCR yielded a product of the expected size of 241 nucleotides; its sequence was identical to that predicted from the genomic and cDNA clones. The full-length open reading frame of Qik with 798 amino acids that add up to a molecular weight of 90 kDa is shown in Fig. 3.

Expression profile of the Qik protein. The relationship between Qik and Qin was further examined by using a chimeric construct, Qin-ER, in which a truncated version of v-Qin was fused to the hormone binding domain of human estrogen receptor (Fig. 4A). Qin-ER, expressed by the avian retroviral vector RCAS, acts as a weak transcriptional repressor and induces cellular transformation, solely in the presence of 4-hydroxy-tamoxifen (4-HT) (data not shown). In the absence of 4-HT, the chimeric Qin-ER protein was detectable by immunofluorescence in the cytoplasm of CEF transfected with RCAS-Qin-ER, addition of 200 nm 4-HT resulted in nuclear translocation of the protein. Qik mRNA levels increased about threefold at 1 h after activation of Qin-ER by 4-HT. CEF transfected with the empty RCAS vector did not respond to 4-HT with increased levels of Qik transcripts. This result suggests that upregulation of Qik is an early downstream event triggered by the Qin protein in transformed cells. Northern blots with total RNA from various chicken embryonic tissues showed ubiquitous expression of Qik. Such broad expression of Qik contrasts with the telencephalon-specific expression of Qin. The basal expression of Qik must therefore be driven by regulators other than Qin.

Qik is a protein kinase with auto-phosphorylation activity. In order to determine whether the Qik protein is an active kinase, CEF were transfected with either RCAS-Qik or RCAS-Qikm. In the latter construct, the lysine at position 55 had been changed to methionine, thus replacing a conserved amino acid required for ATP binding. Cells were harvested after three passages, allowing for spread of the infectious vector. Qik proteins were immunoprecipitated from cell lysates with anti-HA antibody (12CA5), and *in vitro* kinase assays were performed. Several known kinase substrates including histone H1a, H1b and cdc25C were incubated with the immunoprecipitated Qik protein but none was phosphorylated. However, a band at the size of Qik (90 kD) was seen when wild type, but not the mutant Qik alone was tested in the presence of [γ-^{32}P]ATP (Fig. 5A). A control experiment showed that mutant and wild type proteins were expressed at the similar levels (Fig. 5B). This result suggests that Qik can function as a kinase *in vitro* and that it has auto-phosphorylation activity.

Overexpression of qik is not sufficient to cause transformation but is also induced by the src oncogene. The up-regulation of Qik in Qin-transformed cells raised the question whether the newly isolated kinase is also involved in cellular transformation. Although transfection of CEF with RCAS-Qik resulted in overexpression of the Qik protein and increased the rate of cell prolif-

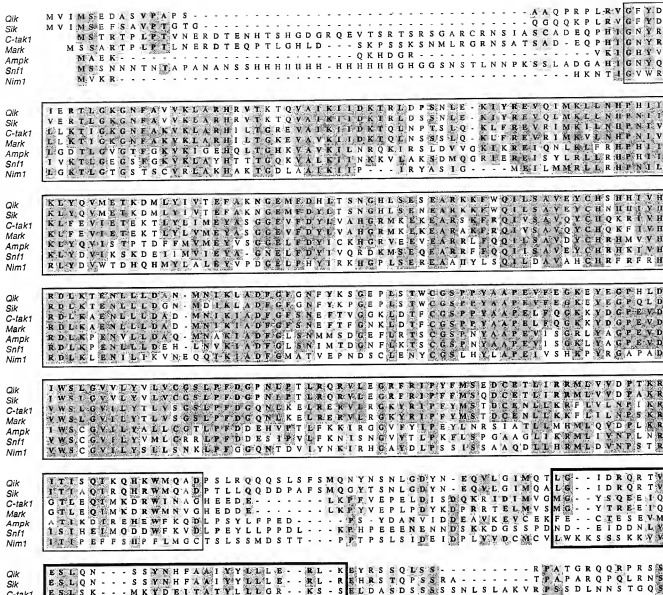

FIG. 2. Sequence comparison of Qik and members of the AMPK/SNF1 family of serine/threonine kinases. The domains were aligned using the CLUSTAL W algorithm. Identical residues and conservative substitutions are indicated by gray highlighting. Sequences boxed by the thin line represent the catalytic domain. Sequences boxed by the heavy line represent the SNH domain that defines the AMPK/SNF1 family.

eration slightly, it induced neither formation of transformed foci nor anchorage-independent growth. Therefore, up-regulation of Qik has only a marginal effect on the growth properties of the cell. CEF in which Qik was overexpressed also did not show enhanced susceptibility to Qin-induced transformation. In order to explore

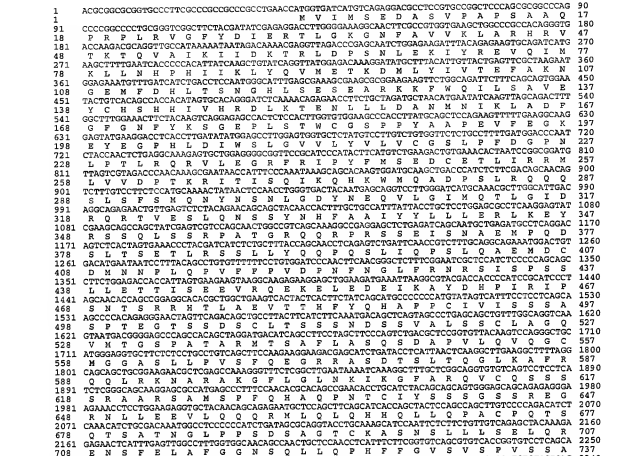

FIG. 3. cDNA and amino acid sequences of Qik. The sequence in the 5′ region (nt 1–60) was derived from a genomic clone. The resultant transcript was confirmed by RT-PCR and sequencing. The sequences shown were deposited in GenBank (Access Domain No. AF219232).

the possibility that Qik expression is responsive to other growth-promotive signals, a Northern blot analysis was carried out with CEF transformed by Src, Jun or P3k (14) (Fig. 6). Elevated levels of Qik mRNA were seen in Src but not in P3k or Jun transformants, suggesting that signal cascades of Qin and Src may share

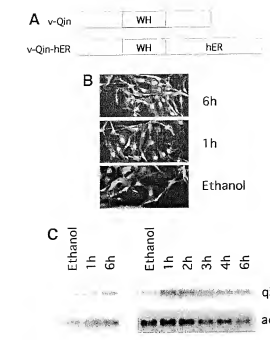

FIG. 4. Expression of Qik in the Qin-ER inducible system. (A) Diagram of the RCAS-Qin-ER construct (hER, hormone-binding domain of the human estrogen receptor, WH winged helix domain). (B) Immunofluorescence of RCAS-Qin-ER-transfected cells. CEF were treated with 200 nm 4-HT for 6 h (top) or 1 h (middle) or ethanol diluent (2 μl in 2 ml culture medium) (bottom) and stained with Qin polyclonal antibody and FITC-conjugated anti-rabbit IgG. (C) Northern blot analysis. mRNA from RCAS (left panel) and RCAS-Qin-ER-transfected (right panel) CEF that had received ethanol diluent, or 4-HT for 1, 2, 3, 4, and 6 h was separated on an agarose gel, transferred to a nylon membrane and probed with Qik or actin cDNA.

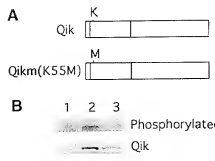

FIG. 5. Autophosphorylation of Qik. (A) Diagram of Qik and Qikm, carrying a lysine to methionine substitution at position 55 in the kinase domain. (B) CEF transfected with RCAS (lane 1), RCAS-Qik (lane 2), or RCAS-Qikm (lane 3) were harvested and immunoprecipitated with a monoclonal anti-HA antibody (12CA5). The product was incubated with [γ^{32}P]-ATP in kinase assay buffer at 30°C for 20 min, separated on SDS–PAGE and transferred to a nitrocellulose membrane for autoradiography (upper panel). The lower panel shows a Western blot with the same cell lysates. Forty milligrams protein from each lysate were separated on a SDS–PAGE and transferred to a nitrocellulose membrane. The blot was incubated with the 12CA5 anti-HA monoclonal antibody and horseradish peroxidase-conjugated sheep anti-mouse serum.

downstream targets or that the upstream regulatory regions of *qik* contain Src as well as Qin responsive elements.

DISCUSSION

Qik belongs to the AMPK/SNF1 family of serine-threonine kinases. These proteins show homology in the catalytic domain and relatedness in a domain called the SNF1 homology region. The latter defines the AMPK/SNF1 family of kinases. Members of the family diverge in non-catalytic sequences that may determine biological functions and confer substrate specificities. The prototypes of the family are AMP-activated kinase (AMPK) and its yeast homologue SNF1. These kinases are conserved among fungi, plants and animals. They are upregulated by AMP, responding to ATP depletion. They set in motion the activation of ATP-producing catabolic pathways and the downregulation of ATP-consuming anabolic pathways. AMPK/SNF1 kinases are stress-induced. They have also been called the fuel gauges of the cell (19, 20). An important member of the family of AMPK/SNF1 kinases is nim1/cdr1 of fission yeast (21, 22). This kinase phosphorylates and thereby inactivates the wee-1 kinase, which is an inhibitor of mitosis. Overexpression of nim1 advances cells into mitosis at a reduced cell size, while the null-mutation of nim1 delays mitosis, resulting in enlarged cell sizes (23). Cdc25-associated protein kinase (C-TAK1) is yet another member of the family linked to multiple cellular functions, including effects on the cell cycle (24). By homology, Qik is most closely related to the salt-inducible kinase (SIK). SIK was isolated from rat adrenal gland and is stimulated by high plasma levels of K^+, Na^+ or by adrenocorticotropic hormone (ACTH) (18). It may play an important role in adrenocortical functions. From this short list it is clear that the AMPK/SNF1 kinases have diverse regulatory roles that include control of cell growth. Qik may follow one of these known models, or it may represent a novel regulatory function.

Qik is an early target of Qin, upregulated very rapidly after Qin activation. However, it is probably not a direct target, because the only detectable transcriptional regulatory function of Qin is repression of target

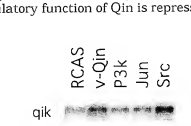

FIG. 6. Expression of *qik* is induced by Src but not by Jun and P3k. mRNA (2 μg/lane) from CEF transfected with RCAS, RCAS-v-Qin, RCAS-v-P13k, RCAS-v-Jun or RCAS-v-Src was analyzed on a Northern blot probed with Qik cDNA. Actin was used as a control.

genes, not activation of transcription. Qik is more likely to be activated directly or indirectly by the Qin-induced downregulation of a repressor. The ubiquitous expression of Qik also suggests that basal expression of this kinase is under the control of other regulators. In a previously described chimeric construct, Qin has been turned into an activator by replacing its native repression domain with the potent transactivation domain of the herpes simplex virus protein VP16. This chimera is nontransforming and induces specific resistance to Qin transformation in CEF (9). As expected, Qin-VP16 downregulates Qik. The expression profiles of Qin- and Src- but not of Jun- or P3k-transformed CEF include upregulation of Qik. Qik is therefore not a universal marker of the neoplastic phenotype. Whether Qik contributes to oncogenic transformation or is a neutral bystander is not known. Experiments with antisense constructs or transdominant negative mutants could answer the question as to whether overexpression of Qik is necessary for transformation. The available data reported in this communication show that upregulation of Qik alone is not sufficient for a substantial change in the growth properties of the cell. Additional target genes of Qin will have to come into play in order to effect transformation.

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